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# In Vitro Synthesis of Infectious Retroviral RNA†

FRÉDÉRIC FLAMANT‡* AND JOSEPH A. SORGE§

*Department of Basic and Clinical Research, Research Institute of Scripps Clinic, La Jolla, California 92037*

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**A plasmid was constructed in which a T7 RNA polymerase promoter was placed upstream of a recombinant amphotropic retrovirus genome containing a selectable neomycin resistance gene. To test the infectivity of the RNA produced by T7 RNA polymerase in vitro, the RNA was microinjected into the nuclei of $\psi_2$ packaging cells. Infectious particles conferring G418 resistance were released.**

Retroviruses are RNA viruses that have the ability to replicate through a DNA intermediate. This is achieved by reverse transcription of retroviral genomic RNA by the virus-encoded reverse transcriptase. A double-stranded DNA provirus with two long terminal repeats (LTRs) is generated from a template of genomic RNA by a complex mechanism. The accumulation of experimental data has led to the proposal of a model for reverse transcription that explains the synthesis of the LTRs (13) (Fig. 1). The wide acceptance of this model relies mostly on in vitro studies of reconstructed systems and detergent-disrupted virions. However, in vivo experiments supporting this model are not as numerous (for a review, see reference 13).

One approach to study this mechanism in vivo would be to create definite mutations in an in vitro-made RNA, which would be reintroduced into the cells to analyze the consequences on the DNA provirus structure. Purified natural retrovirus RNA has already been shown to be infectious after cellular microinjection (10). However, retroviruslike RNA produced in vitro by a bacteriophage polymerase would differ from natural retroviral RNA, since it would not undergo polyadenylation, methylation (4), and perhaps other unknown modifications. Whether all of these modifications are necessary is unknown. Whether they occur in vivo after nuclear microinjection is also uncertain.

In the present experiments, we designed a plasmid that produced a retroviruslike RNA in vitro with T7 RNA polymerase. This synthetic RNA was found to be infectious after microinjection into the nuclei of a packaging cell line, although at a low level. Furthermore, we found that if the 5′-most 30 nucleotides were missing from the in vitro RNA, a full-length DNA provirus was produced in vivo. The natural R region of murine leukemia virus is 69 nucleotides long. This result supports observations regarding R region mutations reported by Lobel and Goff (6).

The plasmid Retroscript was designed for the insertion of most murine retrovirus genomes and the in vitro production of RNA molecules similar in sequence to natural retrovirus RNA. T7 RNA polymerase is ideal for this, because it is highly selective for its own promoters and it efficiently makes complete transcripts from long DNA molecules (1). However, murine retrovirus RNAs usually start with the 5′ sequence GCG, while T7 polymerase transcripts always start with 5′ GGG. Therefore, we decided to start the transcription at retrovirus position +30 (after the cap site), where the first GGG is found in the natural amphotropic retrovirus RNA. As explained below, in vivo reverse transcription of these truncated RNAs is expected to be unaffected by this deletion and to produce a full-length DNA provirus.

Figure 2 summarizes the successive steps of plasmid construction. A double-stranded synthetic oligonucleotide was inserted in the polylinker of pUC118 (a plasmid derived from pUC18 with an M13 origin of replication; gift from J. Messing) between the *Eco*RI and *Kpn*I sites to create a promoter for T7 RNA polymerase. The transcription initiation site of the promoter was designed to coincide with a unique *Kpn*I site. A second double-stranded oligonucleotide, copied from the end of the retroviral R sequence, was inserted just after the *Kpn*I site to the *Hin*dIII site. In the resulting plasmid, called Retroscript, the nucleotide sequence between the two unique *Kpn*I and *Mlu*I sites is identical to the one found at the end of the amphotropic retrovirus 4070A (8). The *Kpn*I site can be used to insert the genome of many murine retroviruses which are cut by *Kpn*I within the LTR R sequences. The *Eco*RI and *Hin*dIII sites are no longer present in Retroscript. We inserted the genome of N2, a replication-defective virus derived from the Moloney murine leukemia virus in which the neomycin resistance gene is placed under the control of the upstream LTR promoter, into the unique *Kpn*I site (5).

The resulting plasmid, $N_2$script, was used to synthesize in vitro RNA molecules similar to $N_2$ genomic RNA but lacking the first 30 nucleotides and a poly(A) tail.

$\psi_2$ and 3T3 cells were grown as monolayers in Dulbecco modified Eagle medium with 10% fetal bovine serum, penicillin, and streptomycin. Resistant cells were selected in the same medium supplemented with the antibiotic G418 at 350 μg/ml. $\psi_2$ is a retrovirus-packaging cell line which allows the production of replication-defective retrovirus vectors in the absence of competent helper virus (7). After microinjection into $\psi_2$ cell nuclei, we expected a normal maturation and packaging of the in vitro-made $N_2$script RNA into viral particles. If these particles can infect a sensitive cell, the packaged RNA is expected to be reverse transcribed and the DNA provirus is expected to be integrated and confer G418 resistance to the infected cell.

Template plasmid DNA was linearized by *Mlu*I digestion, treated with proteinase K (100 μg/ml, 20 min, 37°C) to eliminate contaminating RNase activity, extracted with phenol-$CHCl_3$ (1:1, vol/vol), and precipitated with ethanol. In

* Corresponding author.

† Publication 5079BCR of the Research Institute of Scripps Clinic.

‡ Present address: Laboratoire de Génétique Animale INRA, Ecole Nationale Supérieure Agronomigue, 65 rue de Saint Brieuc, 35042 Rennes Cedex, France.

§ Present address: Stratagene, 11099 North Torrey Pines Road, La Jolla, California 92037.

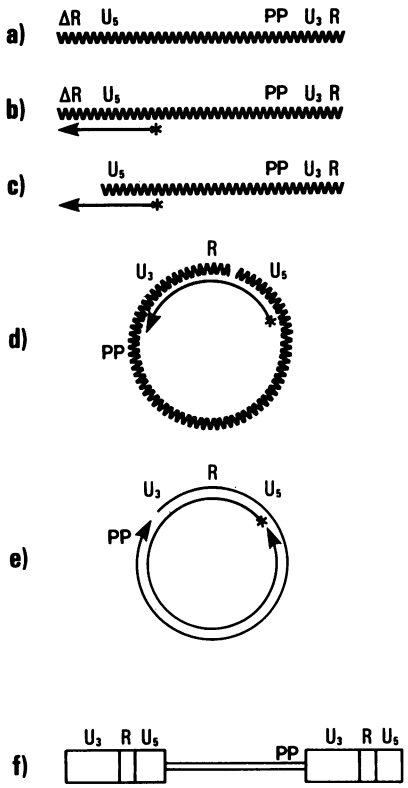


FIG. 1. Production of normal provirus DNA according to the standard model. According to the favored model for retrovirus RNA reverse transcription (13), the RNA synthesized in vitro from $N_2$script should produce a normal DNA provirus after in vivo reverse transcription despite the lack of the first 30 nucleotides in the upstream R sequence (R) (6). The in vitro product of T7 transcription (a) is used as a template for the initiation of minus-strand DNA synthesis (b). The 5′ end of the RNA is then degraded by the RNase H activity (c). The single-stranded protruding DNA end is homologous to the downstream R sequence, which is used as a template to continue the first-strand synthesis (d). Plus-strand synthesis is initiated in the polypurine tract (PP) during the completion of first-strand synthesis (e), and the displacement of the paired sequences allows duplication of the LTR (f). In the resulting DNA provirus, both R sequences are full length.

vitro transcription was initiated with 10 μg of linearized template DNA in 250 μl of a solution containing 50 mM Tris (pH 8), 10 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 0.4 mM ATP, 0.4 mM CTP, 0.4 mM UTP, 60 μM $m^7G(5')ppp(5')Gm$ (Pharmacia, Inc., Piscataway, N.J.), 30 mM dithiothreitol, 100 U of placental ribonuclease inhibitor (Stratagene, San Diego, Calif.) per ml with 100 U of T7 RNA polymerase (Stratagene). The transcription of the capped molecules was continued by adding 0.4 mM GTP (37°C, 1 h). The reaction was stopped by proteinase K treatment (20 min), followed by two phenol-$CHCl_3$ extractions, two $CHCl_3$ extractions, three ether extractions, and ethanol precipitation. The pellet was suspended in 100 μl of filtered $H_2O$ (pore size, 0.2 μm). A sample of this RNA preparation gave a single band after agarose gel electrophoresis and ethidium bromide staining.

$\psi_2$ cells were grown on a glass cover slide and microinjected with RNA as described elsewhere (9). The microinjected cells (about 200) were immediately placed in a 100-mm dish of 3T3 cells ($10^5$ in 7 ml of medium supplemented with 8 μg of Polybrene per μl). Since interference prevents $\psi_2$ cells from self-infection and since DNA provirus integration requires de novo exogenous infection, the $\psi_2$ cells cannot be expected to become G418 resistant. Thus, the microinjected $\psi_2$ cells were cocultivated for 2 days with 3T3 fibroblasts, which were then selected for resistance to G418. After 10 days in selective medium, two G418-resistant colonies were observed.

Electroporation was performed with a suspension of trypsinized $\psi_2$ cells which previously had been rinsed twice with phosphate-buffered saline (0.15 M NaCl, 10 mM phosphate, pH 7.4) in a tube containing 5 μg of in vitro-made RNA per 500 μl of phosphate-buffered saline. A total of $10^3$ cells were submitted to one electric pulse (1,750 V/cm, 50 ms with Transfector 100 [BTX, San Diego, Calif.]). These cells were then plated on $10^5$ 3T3 cells in 7 ml of medium supplemented with 8 μg of Polybrene per μl. After 2 days of cocultivation, the resistant cells were selected with G418. More G418-resistant colonies were obtained after electroporation was substituted for microinjection. When $10^3$ $\psi_2$ cells were electroporated with 5 μg of in vitro-made RNA and plated in $10^5$ 3T3 cells, nine resistant colonies were obtained after G418 selection. Therefore, the efficiencies of both techniques were similar (approximately one colony per 100 $\psi_2$ cells subjected to electroporation or injection).

We expected that resistant cells would result from the infection of 3T3 cells with viral particles bearing the in vitro-synthesized $N_2$script RNA. However, possible experimental artifacts had to be considered carefully. (i) Accidental contamination of the cells by a retrovirus carrying the neomycin resistance gene was ruled out, since we did not grow such a virus in our laboratory at the time of this experiment. (ii) The $N_2$script DNA, used as a template for in vitro transcription, was cointroduced with the RNA into the $\psi_2$ cells. Although this plasmid does not possess any eucaryotic promoter upstream of the neomycin resistance gene, it is conceivable that this plasmid could integrate into the $\psi_2$ cell genome near a chromosomal sequence that would activate the neomycin resistance gene expression. First, we showed that $N_2$script DNA alone was incapable of producing G418-resistant cells by microinjection or electroporation. Also, to address this second possibility, we tested the ability of the G418-resistant cells to produce the $N_2$ virus. Since these cells were obtained in helper-free conditions, we did not expect, and did not find, any spontaneous production of virions by the resistant cells (in 10 ml of supernatant). After superinfection with an amphotropic 4070A helper virus (8), the cell supernatant was then found to contain a high number of viral particles conferring resistance to G418 (about $10^5$/ml; see experimental procedures).

This result clearly demonstrated that the G418 resistance initially observed resulted from the infection of 3T3 cells by virions carrying the in vitro-made RNA and not from random DNA integration in the treated $\psi_2$ cells. We further confirmed our conclusion by a Southern blotting experiment, which showed that the G418-resistant cells had integrated an intact, full-length copy of the $N_2$ provirus DNA (Fig. 3).

As outlined above, the first 30 nucleotides of the retrovirus RNA are missing in the in vitro-made product. According to currently favored models, the deletion should be repaired after one round of in vivo reverse transcription (Fig. 1). To test this prediction, we analyzed, by S1 mapping, the RNA produced by one of the G418-resistant cell clones. The 5′ R sequences were repaired during reverse transcription (Fig. 4). This result is consistent with those of Lobel and Goff (6). We also found that the length of the downstream R sequence was that expected (data not shown).

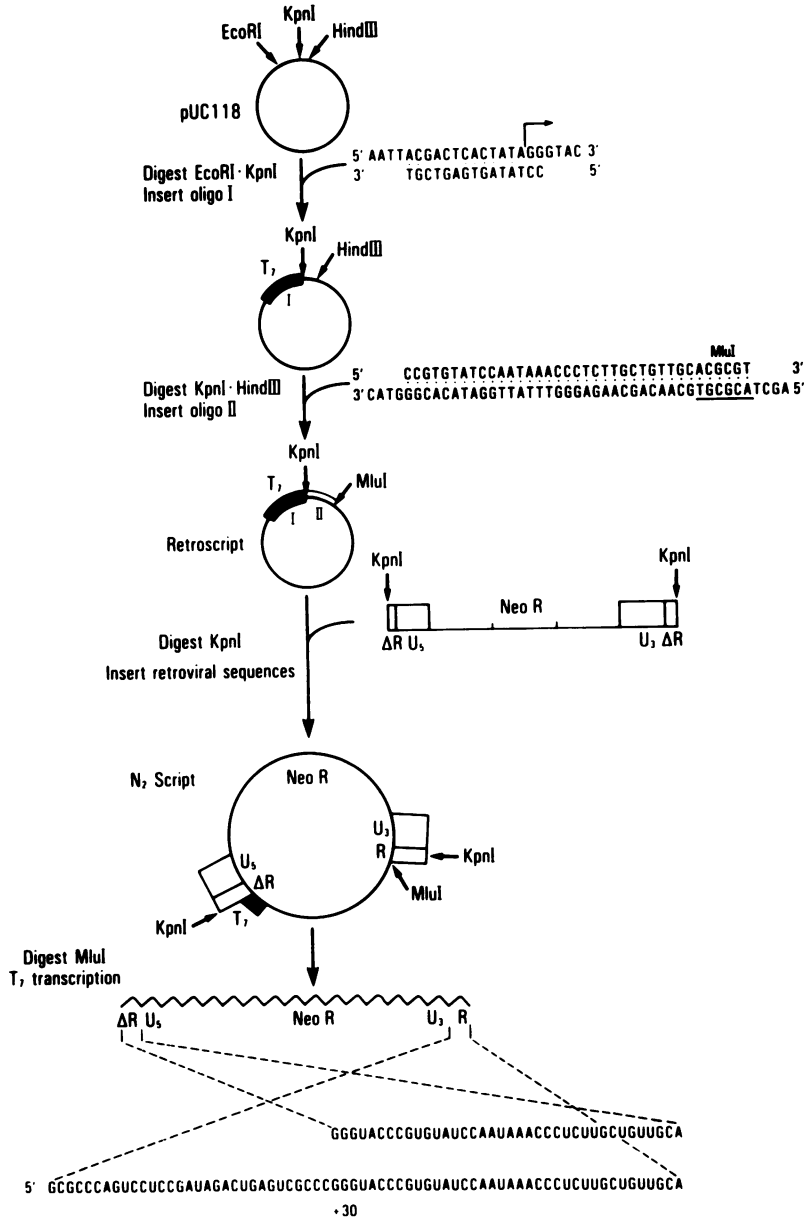


FIG. 2. Construction of $N_2$script. A double-stranded oligonucleotide (oligo I) was inserted between the *Eco*RI and *Kpn*I restriction site in the polylinker of pUC118. This creates a promoter for T7 transcription (the broken arrow indicates the site for the initiation of transcription). A second double-stranded oligonucleotide (oligo II) copied from the end of the retrovirus R sequence was inserted between the *Kpn*I and *Hin*dIII sites, creating a unique *Mlu*I restriction site. This plasmid, called Retroscript, was digested with *Kpn*I to insert a fragment representing the complete genome of $N_2$, a derivative from the Moloney murine leukemia virus carrying the neomycin resistance gene (5). The resulting plasmid, $N_2$script, can be digested by *Mlu*I and used as a template for T7 transcription. As indicated at the bottom of the figure, the 30 first nucleotides of the viral genome are missing from the in vitro product.

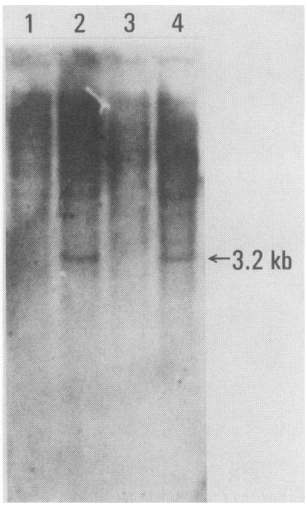


FIG. 3. Hybridizations demonstrating incorporation of $N_2$ DNA provirus. DNA (10 μg) extracted from 3T3 cells (lanes 1 and 3) or from one G418-resistant cell clone (lanes 2 and 4) was digested with *Kpn*I (lanes 1 and 2) or *Sac*I (lanes 3 and 4), electrophoresed, transferred to a nitrocellulose filter, and hybridized to a probe specific for the N2 genome. Both enzymes released a 3.2-kilobase (kb) fragment characteristic of the integrated $N_2$ provirus. Note that the LTR sequences of the $N_2$ probe also cross-hybridized with endogenous proviruses, resulting in the signal above the 3.2-kilobase band.

We have demonstrated here that a retrovirus RNA produced in vitro and microinjected into $\psi_2$-packaging cell nuclei can be encapsidated into infectious virions. After infection, the in vitro-made RNA provided a suitable template for the synthesis of a full-length DNA provirus despite the absence of the first 30 nucleotides. This observation confirms a prediction of the currently accepted model for reverse transcription (6).

Several factors could explain the efficiency of the viral production obtained. Comparisons of our results with those of published experiments in which natural retrovirus RNAs were microinjected (10, 11) are difficult. These published experiments were mainly designed to study the translation of the microinjected RNA revealed by complementation of an *env* mutation in an integrated DNA provirus. However, when a helper genomic RNA was microinjected, both translation and encapsidation were observed. As in our experiments, the encapsidation efficiency was very low (10, 11). A possible explanation could be that the microinjected RNA is efficiently translated and consequently diverted from the encapsidation pathway. We cannot rule out, however, that our in vitro-synthesized RNA is not as highly infectious as a natural retrovirus RNA.

Several modifications of the experimental parameters did not improve the level of virus production, such as polyadenylation of the RNA. Also, another *neo*-carrying vector and another helper cell line performed similarly (8).

This technique could have interesting implications in gene transfer experiments, since a eucaryotic LTR promoter is not needed. A number of problems are linked to the presence of a promoter in the LTR: expression is repressed in embryonal cells (12), cellular flanking sequences can be activated (3), and promoter interference hampers the use of internal promoters (2). The possibility of synthesizing the retrovirus vector RNA in vitro enables the design of gene transfer protocols in which the LTR promoter is entirely unnecessary.

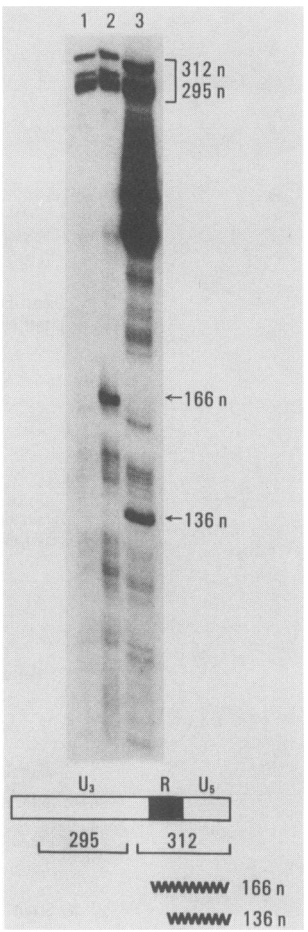


FIG. 4. Analysis of RNA from G418-resistant cells. RNA (30 μg) was extracted from G418-resistant cells (lane 2) or synthesized in vitro from $N_2$script DNA (lane 3). Lane 1 is a control with no RNA. RNAs were hybridized to a 5′-end-labeled probe consisting of a mixture of two fragments covering the upstream part of the LTR (295 base pairs [bp]) and the ends of $U_3$, R, and $U_5$ (312 bp). After S1 nuclease digestion, the hybrids were loaded on a 6% polyacrylamide urea gel. The 5′ ends of the RNAs extracted from G418-resistant cells protected 166 bp of the 312-bp fragment. The in vitro-synthesized RNA protected only 136 bp. Since an excess of in vitro-made RNA was used, incompletely digested products are visible on lane 3. n, Nucleotides.

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## LITERATURE CITED

1. **Chamberlin, M., J. McGrath, and L. Workell.** 1970. New RNA polymerase from E. coli infected with bacteriophage T7. Nature (London) **228**:227–231.
2. **Emerman, M., and H. M. Temin.** 1984. High-frequency deletion in recovered retrovirus vectors containing exogenous DNA with promoters. J. Virol. **50**:42–49.
3. **Hayward, H. S., B. G. Neel, and S. L. Astrin.** 1981. Activation of cellular onc gene by promoter insertion in ALV induced lymphoid leukosis. Nature (London) **290**:475–480.
4. **Kane, S. E., and K. Beemon.** 1985. Precise localization of $m^6A$ in Rous sarcoma virus RNA reveals clustering of methylation sites: implication for RNA processing. Mol. Cell. Biol. **5**:2298–2306.

5. **Keller, G., C. Paige, E. Gilboa, and E. S. Wagner.** 1985. Expression of a foreign gene in myeloid and lymphoid progency of common hemopoietic precursors. Nature (London) **318**:149–154.
6. **Lobel, L. I., and S. P. Goff.** 1985. Reverse transcription of retroviral genomes: mutations in the terminal repeat sequences. J. Virol. **53**:447–455.
7. **Mann, R., R. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
8. **Sorge, J., D. Wright, V. D. Erdman, and A. E. Cutting.** 1984. Amphotropic retrovirus vector system for human cell gene transfer. Mol. Cell. Biol. **4**:1730–1737.
9. **Stacey, D. W.** 1981. Microinjection of mRNA and other macromolecules into living cells. Methods Enzymol. **79**:76–88.
10. **Stacey, D. W.** 1986. Microinjection studies of retroviral polynucleotides. Curr. Top. Microbiol. Immunol. **123**:23–37.
11. **Stacey, D. W., V. G. Allfrey, and M. Hanafusa.** 1977. Microinjection analysis of envelope glycoprotein messenger activities of avian leukosis viral RNAs. Proc. Natl. Acad. Sci. USA **74**: 1614–1618.
12. **Teich, N. M., R. A. Weiss, G. R. Martin, and D. R. Lowy.** 1977. Virus infection of murine teratocarcinoma stem cell lines. Cell **12**:973–982.
13. **Varmus, H. E., and R. Swanstrom.** 1982. Replication of retroviruses, p. 369–512. *In* R. A. Weiss (ed.), RNA tumor viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.